REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
		FINAL 30 Sep 92 TO 29 Sep 95

4. TITLE AND SUBTITLE

CELL CULTURE AND TRANSPLANTATION OF THE SUPRACHIASMATIC CIRCADIAN PACKMAKER

5. FUNDING NUMBERS

F49620-92-J-0517
2312/CS
61102F

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0224

11. SUPPLEMENTARY NOTES

DISTRIBUTION STATEMENT A
Approved for public release
Distribution Unlimited

12a. DISTRIBUTION AVAILABILITY STATEMENT

Approved for public release;
distribution unlimited.

12b. DISTRIBUTION CODE

19970210 012

13. ABSTRACT (Maximum 200 words)

Circadian activity rhythms that have been eliminated by lesions of the suprachiasmatic nucleus (SCN) can be restored by fetal SCN grafts. Partial lesions of the host allow simultaneous expression of both donor and host rhythm. Because partial SCN ablation produces characteristic changes in activity rhythms that are similar to those that occur with age, including shortened period, reduced amplitude, and fragmentation, we investigated the extent to which fetal SCN grafts may be expressed by an animalwhose activity rhythm exhibits these age-dependent changes. The results indicate that expression of a transplanted clock is possible in an unlesioned aged host. Grafts of fetal SCN into young hosts and cortical tissue grafts into intact aged hosts have no effect. In those aged animals that received SCN grafts, three patterns of expression emerged in the subsequent locomotor activity record: complete dominance of locomotor rhythmicity by donor; relative coordination between donor and host rhythms; and spontaneous swithching between host and donor phenotypes. The results suggest that the expression of rhythmicity by the grafted SCN may depend on the relative amplitude or strength of signals produced by the host and donor SCN.

14. SUBJECT TERMS	15. NUMBER OF PAGES
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
(U)	(U)	(U)	(U)

TECHNICAL REPORT

Air Force Office of Scientific Research Grant F49620-92-J-0517

October 1, 1992 - September 30, 1995

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DEGREES CONFERRED

Mark W. Hurd, PhD Psychology, 1996. *Pacemaker-pacemaker communication in the mammalian circadian system.*

Anuradha Mathur, MA Psychology, 1995.

Melo, Lucy, MS Zoology, 1996. *The Role of Nitric Oxide in the Regulation of Circadian Rhythms.*

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Takeuchi, J., Walters, D. and Ralph, M.R. Modification of behavioral responses using double stranded DNA containing target sequences for transcription factors.

Hurd, M.W., Golombek, D.A. and Ralph, M.R. Pacemaker-pacemaker interactions in circadian chimeras.

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Hurd, M.W., Golombek, D.A. and Ralph, M.R. Use of mice carrying a lac-Z transgene marker for analysis of suprachiasmatic cell and tissue grafts in mice

SUMMARY OF RESEARCH

1 Expression of SCN grafts in aged animals (Hurd et al., 1995)

Circadian activity rhythms that have been eliminated by lesions of the suprachiasmatic nucleus (SCN) can be restored by fetal SCN grafts. Partial lesions of the host allow simultaneous expression of both donor and host rhythm. Because partial SCN ablation produces characteristic changes in activity rhythms that are similar to those that occur with age, including shortened period, reduced amplitude, and fragmentation, we investigated the extent to which fetal SCN grafts may be expressed by an animal whose activity rhythm exhibits these age-dependent changes. The results indicate that expression of a transplanted clock is possible in an unlesioned aged host. Grafts of fetal SCN into young hosts and cortical tissue grafts into intact aged hosts have no effect. In those aged animals that received SCN grafts, three patterns of expression emerged in the subsequent locomotor activity record: complete dominance of locomotor rhythmicity by donor; relative coordination between donor and host rhythms; and spontaneous switching between host and donor phenotypes. The results suggest that the expression of rhythmicity by the grafted SCN may depend on the relative amplitude or strength of signals produced by the host and donor SCN.

2 Restoration of host rhythmicity following SCN transplantation (Hurd dissertation, 1996; manuscript in preparation)

Wheel running appears to be a motivated behavior that is regulated by at least two processes: (1) a circadian clock that sets a window during which the behavior is performed; and (2) a mechanism for regulating the amount of activity within the window (Osiel et al., 1996). The first of these involves the circadian pacemaker cells in the SCN. We have now begun to investigate whether the second is also a property of this nucleus.

To approach this question, we have produced circadian chimeras using aged hosts carrying SCN implants from different tau genotypes. The preparation allows us to determine whether there is a change in the host rhythm that is not attributable to the expression of a new rhythm *per se*.

Restoration of host rhythmicity, including an increase in rhythm amplitude and total amount of activity per day or cycle, occurred following SCN transplantation but not following cortex or sham implantation's. The changes did not depend on the overt expression of a second rhythm, in fact in some cases donor-determined periodicity was not detectable using either computer or subjective visual analysis.

The data from all of these studies indicate therefore that SCN implants may have three distinct effects on he behavior of the host (1) expression of a second rhythm (2) period modulation of the host rhythm; and (3) regulation of the amplitude of the host rhythm.

The last of these suggests that trophic factors may be responsible for maintaining robust rhythmicity in younger individuals. These may be reduced or lost with aging and restored with the fetal SCN graft.

3. Relationship to longevity (Hurd dissertation, 1996; manuscript in preparation).

A study of our breeding colony in Toronto revealed that when animals are housed in constant conditions (dim LL), the average life span for the three tau genotypes is not significantly different. However, when the colony has been maintained under LD 14:10, the life span of the heterozygous tau mutants is significantly shortened compared with both homozygous genotypes and compared with heterozygotes under LL conditions.

Although our outbreeding program should randomize genetic backgrounds, we examined the potential influence of genetic background on a set of F2 litters housed under LD conditions. Animals were housed individually and were undisturbed except for cage changes performed by technicians who were blind to the genotypic conditions. The results recapitulated the colony data: the average life span of heterozygous mutants was shorter than either homozygous groups, but the variance was essentially the same.

At this point it is not clear what factors are responsible for the reduced longevity of heterozygotes under LD conditions; however, we hypothesize that a causal factor is the disruption of robust circadian organization of physiology and behavior that is reflected in the locomotor records. To test this hypothesis, we have analyzed the post-operative survival of aged hamsters that have been given SCN implants.

When behaviorally aged animals are used as hosts, roughly half do not survive longer than 1 month following surgery. This is the same for experimental and control groups. Of those that survive longer, the post-operative longevity is significantly greater for SCN recipients than for the other groups. These findings support the hypothesis that the organization of physiology and behavior provided by the SCN is a factor in determining the health and longevity of the individual.

4. Glia implantation

The many distinct sub populations of cells within the SCN suggest that each may have their own peculiar roles in rhythm generation and control. A reasonable candidate for producing improvements in host-generated rhythmicity are the astroglia, which regulate neuronal activity through numerous mechanisms.

To test this specific role for these cells, Type I astroglia were grown from SCN explants until all evidence of live neurons was eliminated. About 10% of the cultures are other non-neuronal cells (e.g.. endothelial cells) using our methods. In two pilot studies wild type SCN glia were grown in culture for 3 weeks, then pelletized and implanted near the SCN of an intact, aged host (tau mutant). Both the amount of activity per cycle and the amount of time on the wheel were increased following surgery.

5. Nerve growth factor and circadian rhythms in hamsters

5.1 NGF-induced phase shifts of hamster circadian rhythms

Injection of NGF (20 ng/2 μl saline) into the third ventricle adjacent to the SCN caused phase shifts of the locomotor activity rhythms of hamsters held in constant dark conditions (DD). The magnitude and direction of the phase shifts depended on the circadian time of administration in a manner that resembled phase-dependent responses to light pulses. NGF caused significant phase delays at CT 13.5 and phase advances at CT 18, whereas injections at CT 6 and CT 24 had no effect. The average responses, -36 ± 4.7 minute for delays and +56 ± 10 minutes for advances, were approximately half of the amplitude that would be expected from pulses of bright light (cf. Takahashi et al., 1984). However, the phase advance response was not increased at 200 ng NGF (average phase shift = +46.75 ± 1.8 min), suggesting that the maximal effect on the circadian system had been obtained.

5.2 Interactions between light and NGF

Because exogenous NGF induced phase shifts with the same direction and dependence on circadian phase that would be predicted for pulses of light, the possibility exists that the peptide plays a role either in mediating the response to light or modulating sensitivity to photic stimulation. Light pulses (10 lux) administered at CT 13.5 induced phase delays in the wheel-running rhythms of -66 ± 13 min, that were similar in magnitude to those reported previously for the hamster (cf. Takahashi et al., 1984). Central administration of 20 ng NGF prior to the light pulse did not affect the magnitude of the phase shift induced by light alone (-60 ± 6.9 min), although NGF alone induced significant shifts in the same direction (-40 ± 8 min).

5.3 NGF- and light-induced Fos expression at CT 18

The simple, linear cascades of biochemical events leading to NGF- and light-induced phase shifts suggested by the current hypothesis places the gene encoding NGF as a target of light-responsive AP-1 transcription factors. Another possibility is that NGF produces phase shifts by activating a cascade that converges with the photic entrainment pathway. One candidate

protein that may be common to both pathways is the transcription factor, CREB, which is phosphorylated, *in vivo*, in the SCN following light pulses (Ginty et al., 1993) and also in rat PC12 cells following stimulation by NGF (Ginty et al., 1994). Since pCREB is a regulator of AP-1 transcription factors such as c-*fos* and *jun*-B, we determined whether phase-shift inducing concentrations of NGF would also induce IEG expression in the SCN. Light pulses at CT 18 resulted in a strong induction of Fos in the SCN. NGF injections at the same circadian time induced Fos expression (in the SCN area), though not as densely as with a light pulse. Animals that were kept in the dark and injected with vehicle at CT 18 had no Fos expression in the SCN.

6. Effects of a $p75^{NGFR}$ null mutation in mice (Golombek et al., 1995)

The low affinity NGF receptor, $p75^{NGFR}$, is expressed densely in the SCN. Since the administration of NGF in hamsters produced phase shifts that were similar to photic responses, this receptor may be part of the pathway mediating responses both to NGF and to light. To test this, we examined the circadian system of gene-targeted mice carrying a null mutation at the $p75^{NGFR}$ locus (Lee et al. 1992).

Significant differences were found between homozygous mutants and wild type controls when animals were exposed to 24 hour entraining light cycles (14 hours light: 10 hours dark). In a low intensity LD cycle (L=10 lux: D=0 lux), wild type controls performed all of their wheel running behavior in the dark as expected from nocturnal rodents. Mutants synchronized their behavior with the LD cycle but commenced running activity 3-4 hours earlier while the light was still on. Variability among individual freerunning periods did not account for this difference since period was often identical for animals that responded very differently to the LD cycle.

The entrainment results suggest that either the sensitivity or responsiveness of the system to light was reduced in the mutant mice. The altered phase angle of entrainment suggested therefore, that phase delays might be reduced. To test this, mutants and control animals were placed in DD for 10 days before being exposed to a 15 minute pulse of light at CT 15, the point of maximum response determined previously for the mouse (Schwartz and Zimmerman, 1990).

Low intensity pulses (10 lux) at CT 15 produced large phase delays in control animals with magnitudes that were comparable to those reported previously for various strains of mouse. Phase shifts were significantly smaller in the mutant animals at this intensity. At a higher intensity (700 lux) the responses of the mutants and controls were not different from each other. The wild type response was similar at the two intensities.

7. Regulation of circadian photic responses by transcription factors

Much of the efforts expended over the past few years by a number of laboratories has been directed at determining how immediate early genes fit into the biochemistry of rhythm generation and pacemaker responses to external stimulation. Environmental light produces phase shifts of freerunning rhythms in rodents, that are accompanied by, and temporally correlated with, the induction of IEGs. The current data suggest that one or more of these IEGs is part of the transduction pathway that leads to phase shifts following photic stimulation to the retina. However, these data are for the most part correlational, and the only direct evidence for a central role in the transduction pathway comes from experiments where antisense oligonucleotides have been used to inhibit the activity of the AP-1 components, *c-fos* and *jun-B* (Wollnick et al., 1995).

Given that our work with the *c-fos* knockout has shown that at least FOS is not an absolute requirement for light-induced phase shifts, it is hypothesized that other related proteins are sufficient to enable the AP-1 activation of second tier genes. Either this, or alternate pathways are activated in response to light.

To test for the involvement of AP-1 activity itself, we designed false-target DNA sequences (36mer, double stranded) that contained 2 AP-1 target sequences, and applied these directly to the SCN prior to phase shifting light pulses. The assumption here is that the AP-1 complex will bind to the false-target DNA preferentially, thus reducing its effect on the target genes.

The false-target DNA for AP-1 (ftDNA(AP-1)) significantly attenuated photic responses. A control oligonucleotide that was designed with the AP-1 target sequence shuffled (ftDNA(CAP-1) had no effect. Similarly, a second control that contained SP-1 sequences (ftDNA(SP-1)) had no effect. Together, these data confirm the notion that AP-1 activity is required for normal circadian responses to light.

Furthermore, the results demonstrate an approach that has not been attempted before that will now be used to detect the presence of additional transcription factors that can be isolated and identified. Preliminary results using a second shuffled AP-1 target illustrate the potential of this approach. In the course of performing these experiments, a second control oligonucleotide was produced with the idea that we may be able to produce ftDNAs with greater or lesser efficacy than the one already shown to be effective. Surprisingly, the first of these produced the opposite effect than that expected: phase shifts are significantly potentiated by the application of this DNA. The sequence does not correspond to anything that is currently on file.

There are three distinct possibilities. (1) The new sequence has a toxic effect on the pacemaker, unlike the other same size molecules tested. (2) The new sequence binds AP-1 to a greater or lesser extent, and this accounts for a very different change in the system. (3) The new sequence is recognized by an unknown transcription factor.

Experiments now underway are intended to identify the protein(s) from the SCN that are able to bind to the different oligonucleotides.

8. Regulation and integration in the mammalian circadian system

8.1 Role for calmodulin (CaM)

Roles for CaM and CaM regulated processes in the light input pathway were examined in a series of pharmacological studies. CaM activity itself was blocked using a specific inhibitor, W-7 which produces "non-photic" phase shifts when administered alone (Shibata and Moore, 1994). W-7 produced a 50% block of the light response (Golombek and Ralph, 1995). Inhibition of the CaM-dependent kinase II using another relatively selective inhibitor, KN-62, also produced an incomplete block of the light response (Golombek and Ralph, 1994).

These results suggested that other pathways, both CaM dependent and independent may also be activated or necessary for the photic response. This led to the investigation of an NGF signaling pathway since the *ras/raf* transduction pathway is also regulated by calmodulin. These results have been discussed.

8.2 Role for protein phosphatase 2B (calcineurin)

A third CaM-dependent pathway includes the protein phosphatase 2b (PP2B; calcineurin). This pathway has been studied extensively for its role in

activating T-lymphocytes during immune responses. The immunosuppressants, cyclosporin A (CsA) and FK-506, work through the inhibition of calcineurin, although their sites of action are on different proteins. CsA binds directly to a cyclophilin (cyp), and FK-506 to an FK binding protein. These in turn combine with calcineurin to inhibit the CaM-activated enzyme.

We have found that pretreatment with either drug attenuates photic responses to the same extent (60-70%); and CsA produces "non-photic" phase shifts when administered alone. The results, suggesting a potential feedback connection from the immune system (which is regulated by the circadian system) have been submitted to *Nature*.

8.3 Role for cGMP and protein kinase G (PKG). (Mathur et al., 1996).

A role for PKG in light transduction was investigated using inhibitors of PKG, PKA and PKC. Only agents that are known to inhibit PKG had any effect on light-induced phase shifts. Significantly, the effect was obtained only at CT18 where light induces phase advances, and not at CT13.5 where light-induces delays. These results have been obtained by other who have suggested that different biochemical pathways underlie phase shifting at the two time points. In contrast, we interpret these results as suggesting that the drugs are acting on a target system that is rhythmic; the same biochemistry is involve at both time points, but the degree to which it is involved depends on the prior environmental history of the individual.

8.4 Role for nitric oxide and nitric oxide synthase (NOS). (Melo et al., submitted)

Because cGMP/PKG activity is sensitive to NO^-, the activity of this enzyme was examined. We have found that an inhibitor of NOS, L-NAME, produces an attenuation of photic responses at CT18 but not at CT 13.5. This is consistent with the PKG findings described above, but is in contrast with other reports that show a block at both time points. We suggest that this difference is due to subtle differences in the baseline handling of animals between labs.

In addition, we found that the NOS substrate, SNAP, potentiates the response to light. In fact, the response in the delay region is about twice the maximum that can be obtained with light alone.

The results from the SNAP experiment are the first to indicate that NOS plays any role in circadian rhythm regulation other than as a modulator of synaptic transmission.

8.5 Role for c-fos (Honrado et al., 1996).

As discussed above, a major focus of attention in the last few years has been on the role that IEGs play in the regulation of circadian rhythms. To test specifically, the involvement of FOS in light-induced phase shifting, we examined the circadian system of mice that were lacking the *c-fos* gene. A line of mice carrying a null mutation at this locus, produced by homologous recombination (Johnson et al., 1992) was examined for changes in numerous behavioral parameters. We found no significant effect on the ability of these animals to

produce circadian rhythms, and to a first approximation, the responsiveness to light was intact. This is despite the fact that the optic nerve and chiasm was extremely reduced in size. There was an attenuated response to light; however, which could not be overcome with longer or brighter light. This indicated that although FOS was not an absolute requirement for light-induced phase shifting, it was involved in the normal response to light.

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